

Amendments to the Specification:

Please replace paragraph **[0003]** with the following amended paragraph:

**[0003]** This invention relates to the field of molecular biology. In particular, the invention relates to methods and compositions of matter for promoting stable, site-specific integration of Rep-deleted recombinant adeno-associated virus (rAAV) vectors via delivery of a functional AAV Rep gene product to the necessary location by fusing a nucleic acid encoding it to a nucleic acid encoding an intercellular trafficking "cargo" protein such as herpes simplex virus (HSV) tegument protein, VP22 or fragment thereof.

Please replace paragraph **[0008]** with the following amended paragraph:

**[0008]** Accordingly this invention provides a method for mediating site-specific integration of a rep-deleted rAAV vector in a cell, which comprises contacting the cell or expressing ~~on~~ in the cell a fusion polypeptide which comprises an AAV2 Rep protein sequence of the left open reading frame of the rep gene that lacks a functional nuclear localization signal (NLS) and a

VP22 polypeptide sequence that confers intercellular trafficking on the fusion polypeptide. The Rep protein may be fused at the carboxyl or amino terminus of the VP22 polypeptide and may be fused to it directly or indirectly, via a spacer of one or several amino acids. The AAV Rep protein preferably is truncated to remove amino acid residues 489, 490, 491 or 492 and the remaining carboxyl terminus of the translated Rep protein. The truncation most preferably is located at amino acid 490 or 491. ~~DNA constructs and fusion~~ Fusion proteins as described and DNA constructs encoding them also form part of this invention. The invention also provides, in another embodiment, a method of increasing the level of integration of a rAAV vector in a cell comprising contacting the cell with a Rep fusion protein having a mutation in the AAV2 NLS.

Please replace paragraph **[00011]** with the following amended paragraph:

**[00011]** ~~Figure 3 is a cartoon~~ Figures 3A and 3B are cartoons showing progressive carboxyl terminal deletions into the AAV2 Rep nuclear localization signal constructed using polymerase chain reaction and fused to the amino terminal portion (3A) or carboxyl terminal portion (3B) of VP22.

Please replace paragraph **[00014]** with the following amended paragraph:

**[00014]** Figure 6 6A is a flow chart showing the scheme for the analysis of intercellular protein trafficking using flow cytometry. Figure 6B shows results of the flow cytometry analysis.

Please replace paragraph **[00015]** with the following amended paragraph:

**[00015]** ~~Figure 7 presents~~ Figures 7A-7E present FACS analysis of trafficking of the AAVRep<sub>490</sub>VP22 fusion protein.

Please replace paragraph **[00016]** with the following amended paragraph:

**[00016]** ~~Figure 8 is~~ Figures 8A and 8B are a pair of photomicrographs of 293 cells stained with a fluorescein isotriocyanate (FITC)-conjugated antibody directed against the recombinant VP22(Gly)<sub>7</sub>AAV2Rep<sub>491</sub> protein showing VP22(Gly)<sub>7</sub>AAV2Rep<sub>491</sub> trafficking (A) and a DAPI stain to show all cells in the field (B).

Please replace paragraph [00021] with the following amended paragraph:

[00021] Recently, a variety of peptides and proteins, such as the herpes simplex virus tegument protein VP22, have been shown to traffic intercellularly, both as native forms and as fusions with other proteins. See, for example, U.S. Patent No. 6,251,398. This invention takes advantage of this ability to deliver a functional AAV gene product to cells to promote site specific ~~RAAV~~ rAAV integration and gene delivery.

Please replace paragraph [00023] with the following amended paragraph:

[00023] The ability of these cargo proteins to deliver functional genes was used in the present invention to promote site-specific ~~rAAV~~ integration of the nucleic acid encoding rAAV and to increase the level of integration, to significantly enhance the potential safety of the gene delivery and to provide an improved method for expression. A variety of exemplary RepVP22 fusion constructs were constructed in which a nucleic acid encoding AAV rep or fragments thereof were linked in frame to a nucleic acid encoding the N- or C- terminus of VP22 within an expression plasmid (Invitrogen). These constructs were

transfected into 293 cells, where protein expression, intercellular trafficking, and Rep function were monitored. These fusion constructs, for example AAV2Rep<sub>490</sub>VP22, VP22(Gly<sub>4</sub>)-AAV2Rep<sub>491</sub> and VP22(Gly<sub>7</sub>)-AAV2Rep<sub>491</sub>, are considered part of the present invention. See ~~Tables I and III-IV below and Figure 3~~ Table I, Figures 3A and 3B, and SEQ ID NOS:21 and 22.

Please replace paragraph [00028] with the following amended paragraph:

**[00028]** Western analysis demonstrated that all RepVP22 constructs were expressed as stable protein products of expected size. ~~Full~~ Protein expressed from full length rep fused to VP22 did not traffic intercellularly (data not shown). A protein encoded by a fusion gene truncated at nucleotide the nucleotides encoding amino acid 490 of the AAV2 rep gene ~~sequence~~ product did traffic intercellularly as assessed by immunofluorescence microscopy and flow cytometry. See, for example, Figure 7. In this construct, Rep<sub>490</sub>-VP22, the ~~Rep~~ rep open reading frame, truncated at amino acid residue 490 of the translated Rep protein, was fused in frame to DNA encoding the amino terminal end of VP22. Interestingly, an analogous VP22-Rep<sub>491</sub> fusion protein did not traffic. Insertion of DNA encoding 4 and 7

glycine spacers to separate the VP22 and Rep<sub>491</sub> domains and circumvent potential steric hindrance to intercellular trafficking restored the ability to traffic intercellularly. See Figure 5. One of skill in the art will readily recognize that any amino acid residue may be used as a spacer provided that the goal of reducing steric hindrance can be achieved. Therefore, spacer amino acids with small side groups are preferred.

Please replace paragraph [00030] with the following amended paragraph:

**[00030]** To confirm ~~rAAV~~ integration of the nucleic acid encoding rAAV into the AAVS1 site by Rep<sub>490</sub>VP22, PCR products containing vector-cell junction fragments were cloned and sequenced. See Figure 11. Fusion proteins were constructed with His tags to facilitate their isolation and purification. The fusion proteins were assessed for their ability to promote site specific ~~rAAV~~ integration of the nucleic acid encoding rAAV by simply applying them to cells in the form of purified Rep-VP22 fusion proteins.

Please replace paragraph **[00031]** with the following amended paragraph:

**[00031]** To exploit the ability of the fusion cargo proteins to deliver functional protein domains intercellularly, the wild type and several modified AAV2 Rep gene constructs were fused in frame to the nucleic acid encoding VP22 ORF both in amino- and carboxyl-terminal orientations. The fusion proteins were expressed using the highly active CMV IE promoter. Although fusions of VP22 with full length AAV2 Rep did not appear to traffic, specific Rep fusion proteins in which the NLS was truncated, for example VP22(Gly<sub>7</sub>)-AAV2Rep<sub>491</sub>, trafficked intercellularly and were capable of promoting site specific integration of recombinant RAAV vectors. See Figure 8.

Please replace paragraph **[00032]** with the following amended paragraph:

**[00032]** Fusion proteins according to the invention can be expressed by plasmid DNA transfection according to any method known in the art, including calcium phosphate coprecipitation, for example. Once expressed, the fusion proteins traffic to surrounding cells via the VP22 or other intercellular trafficking protein moiety, and can mediate rAAV vector site specific

integration via the AAV Rep moiety. Those of ordinary skill in the art are familiar with such methods and are able to make modifications as desired depending on the protein fusion and cell type(s) involved. Alternatively, fusion proteins can be expressed within cells by introducing expression plasmid DNA via physical methods (lipofection, electroporation, etc.) or by using a viral vector. In addition, purified fusion protein may be applied directly to cells to promote site-specific rAAV vector integration. Because the constructs preferably express fusion proteins with His tags (which allow easy purification by nickel column chromatography) the proteins may be purified after production in bacteria or eukaryotic cells, and then applied directly to cells at the time of rAAV vector transduction. This increases the frequency of rAAV vector integration.

Please replace paragraph **[00033]** with the following amended paragraph:

**[00033]** Plasmids pVP22/myc-His and pVP22/myc-His-2 were obtained from Invitrogen (Carlsbad, CA). See Figures 1 and 2. The nucleic acid encoding the full length AAV2 rep gene product was amplified by PCR and inserted into the pVP22/myc-His vector as an EcoRV and XbaI fragment. The nucleic acid encoding the



AAV2 Rep68/78 open reading frame was amplified from pTZAAV, a pUC-based phagemid containing the full length, infectious AAV2 genome inserted as a Bgl II fragment.

Please replace paragraph **[00034]** with the following amended paragraph:

**[00034]** To construct a VP22-Rep fusion protein with the full length AAV2 Rep, see Figures 1 and 2, the rep gene was amplified as a 1.9 kb fragment using the sense primer 5' GGGAGGTTT**GATATC**GCAGCCGCCATGCCGGGG 3' (SEQ ID NO:1) with incorporation of an Xba I site (bold) and the antisense primer 5'GATTTAAT**TCTAG**ATATTGTTCAAAGATGCAG 3' (SEQ ID NO:2) with incorporation of an Xba I site (bold). The rep mRNA stop codon was modified from TAA to TAT as a part of the Xba I site to permit read-through incorporation of myc and His tags at the 3' end of the fusion protein.

Please replace paragraph **[00035]** with the following amended paragraph:

**[00035]** The 5'-PCR primer used for the construction of the nucleic acid encoding the full length Rep-VP22 fusion protein was 5' GGGTTGAACGCGCA**GATATC**ATGCCGGGG 3' (SEQ ID NO: 3) which

incorporated an EcoRV site (bold). Two different full length Rep-VP22 nucleic acid constructs were made: (1) RepVP22cys, in which the stop codon of ~~Rep~~ the Rep mRNA was modified to a Cysteine residue to allow read-through of the nucleic acid encoding VP22 and (2) RepVP22phe, in which the nucleotides encoding amino acids 620 and 621 were eliminated and the nucleotides encoding residue 619 ~~was~~ were modified from a nucleotides encoding phenylalanine to a nucleotides encoding cysteine to allow for read-through of the nucleic acid encoding VP22. The downstream primer for the RepVP22cys construct was 5' GCCATACCTGATTTA**GCGGCCGC**ATTGTTCAAAGATG 3' (SEQ ID NO: 4), while the downstream primer used to generate the RepVP22phe protein was 5' GATTAAATCATTTA**GCGGCCGC**AGATGCAGTCATCCAAA 3' (SEQ ID NO: 5). Both primers incorporated a Not I site (bold) for cloning purposes. See Figure 3.

Please replace paragraph **[00036]** with the following amended paragraph:

**[00036]** ~~Progressive Fusion proteins containing progressive~~  
carboxyl terminal deletions into the AAV2 NLS protein were  
~~constructed using polymerase expressed from corresponding nucleic~~  
acids encoding the proteins with the terminal deletions.

Polymerase chain reaction and fused was used to fuse the nucleic acids encoding these terminally deleted AAVs NLS proteins to the nucleic acid encoding the amino terminal portion (Figure 3A) or the nucleic acid encoding the carboxyl terminal portion (Figure 3B) of VP22. Initial studies indicated that the AAV2REP<sub>490</sub>-VP22 fusion protein trafficked between cells, but that the corresponding VP22-AAV2REP<sub>491</sub> did not. To circumvent possible steric interference with trafficking, nucleotides encoding 4 and 7 glycines were inserted in frame in the mRNA between the VP22 and AAV2REP<sub>491</sub> open reading frames. All constructs were sequenced to ensure that no mutations were inadvertently introduced following PCR amplification.

Please replace paragraph **[00038]** with the following amended paragraph:

**[00038]** pVP22-Rep constructs ~~with~~ that coded for truncations in the NLS protein were constructed in a similar fashion to the previously described full length rep constructs. Nucleic acids encoding Rep proteins truncated at the carboxyl end at amino acids 484 (VP22AAVRep<sub>484</sub>), 491 (VP22AAVRep<sub>491</sub>), and 519 (VP22AAVRep<sub>519</sub>) were generated by PCR cloning. For these modified proteins, the 5' end of the rep mRNA open reading frame was

amplified with the same sense primer as VP22-Rep  
(5' GGGAGGTTT**GATATC**GCAGCCGCCATGCCGGGG 3'; SEQ ID NO: 1) and  
incorporated an EcoRV site (bold). The 3' end of the Rep mRNA  
ORF for 484, 491 and 519 truncations were amplified with  
antisense primers, 5' GGCTCCACCCTTTT**TGCTAGAA**ATTCATGCTCCAC 3'  
(SEQ ID NO: 6), 5' GGGGGCGGGTCTT**TCTAGA**GCTCCACCCTTTT**TG** 3' (SEQ ID  
NO: 7), and 5' GTTGATCGAAGCT**TCTAGA**TCTGACGTCGATGG 3' (SEQ ID NO:  
8), respectively, all of which incorporated an Xba I site (bold).

Please replace paragraph [00039] with the following amended  
paragraph:

**[00039]** For VP22(Gly)<sub>4</sub>AAVRep<sub>491</sub> and VP22(Gly)<sub>7</sub>AAVRep<sub>491</sub>  
constructs, the 5' end of Rep mRNA ORF was amplified with  
5' CCATTTTGAAGC**GATATC****GGTGGAGGCGGA**GCCGCCATGCCGGGG 3' (SEQ ID NO:9)  
and 5' GGGTCTCCATTT**GATATC****GGGGGGGGTGGAGGCGGAGGC**GCCATGCCGGGG 3' (SEQ  
ID NO:10), respectively. EcoRV sites are in bold while bases  
encoding the glycine spacer residues are in bold and italicized.  
For the 3' end, the antisense primer for the pVP22-Rep491 protein  
mRNA SEQ ID NO:7) was used. The amplified products were digested  
with EcoRV and XbaI, and inserted into similarly digested  
pV22/myc-His. Two nucleic acid constructs encoding full-length

RepVP22 protein and three nucleic acid constructs encoding  
truncated RepVP22 ~~constructs~~ protein were generated.

Please replace paragraph [00040] with the following amended  
paragraph:

[00040] Three ~~truncated Rep constructs~~ nucleic acid constructs  
encoding truncated Rep proteins, AAVRep<sub>469</sub>VP22, AAVRep<sub>490</sub>VP22 and  
AAVRep<sub>505</sub>VP22, were created using independent Not I site-  
containing downstream primers coupled with the identical primer  
used to generate the full-length construct. The AAVRep<sub>469</sub>VP22  
3' primer, 5' GATCCTTTGCCAG**GCGGCCGC**CAGTCTTTGACTTCCTGCTTGG 3' (SEQ  
ID NO:11) extended from +1385 to +1425 with base changes at  
+1405 to +1408 and +1412. These sequence changes in the primer  
modified residue 469 of the expressed protein from a  
phenylalanine to a cysteine and eliminated the production of all  
amino acids C-terminal to residue 469.

Please replace paragraph [00041] with the following amended  
paragraph:

[00041] AAVRep<sub>490</sub>VP22 C-terminal primer,  
5' GGTCTTTT**GCGGCCGC**CACCCTTTTGG 3' (SEQ ID NO:12), extended from  
+1457 to +1483. Mismatches at +1469, +1471, and +1473 to +1475

were used to eliminate all residues C-terminal to 490 in the expressed protein. AAVRep<sub>505</sub>VP22 3' primer, 5' GACTCGCGCAC**GCGGCCGC**GCTCACTTATATCTGCG 3' (SEQ ID NO:13), extended from + 1496 to +1531. It contained nucleotide changes at positions +1513, +1515 to +1517 and +1520 resulting in the loss of amino acids C-terminal to residue 505 in the expressed protein. Additionally, residue 505 in the expressed protein was modified from a proline to an arginine. All C-terminal primers above are given in the reverse orientation. Not I sites are indicated in bold.

Please replace paragraph **[00042]** with the following amended paragraph:

**[00042]** The ~~Rep protein~~ sequence of the Rep<sub>491</sub> truncated ~~construct~~ protein ends at amino acid 491 of the translated Rep protein, however there are 8 amino acids ~~at the junction leading to the initiation codon~~ intervening between the C-terminal of the Rep<sub>491</sub> truncated protein and the initial amino acid of the VP22 polypeptide sequence. These amino acids (DIQHSGGR; SEQ ID NO:14) result from the expression of additional nucleotides found within the multiple cloning site in the vector. Therefore, it is clear to one of ordinary skill that multiple variations of the fusion

peptides are possible, depending on the exact construction methods used to create them. The two moieties of the fusion polypeptide may be fused directly or indirectly, with additional amino acids present at the junction or either terminus. See Table II, below for exemplary sequences contained in the Rep fusion polypeptides compared to Rep wild type. All constructs were analyzed by DNA sequencing to insure that no additional mutations were inadvertently incorporated during the PCR amplifications. See ~~Tables III-VI~~ SEQ ID NOs: 21-24 and Table I for sequence information for exemplary constructs.

Please replace paragraph **[00044]** with the following amended paragraph:

**[00044]** Figure 12 shows a map of CWRHIVAPAP. This construct contains one expression cassette encoding an antisense RNA complementary to the HIV TAR region under RSV LTR control, and another cassette encoding an antisense RNA complementary to the hu-placental alkaline phosphatase (hu PLAP) under PGK promoter control. CWRPGKH is similar to CWRHIVAPAP except for substitution of a PGK hygromycin resistance cassette for the PGK PLAP cassette.

Please replace paragraph [00045] with the following amended paragraph:

[00045] African green monkey Vero (#CCL-81) cells, 293 cells, COS cells and a Detroit 6-derived cell line, 7374, which contains integrated wild type AAV2, were maintained in high glucose Dulbecco's MEM (DMEM) with 2 mM glutamine and 10% heat inactivated fetal calf serum, at 37°C in 5% humidified CO<sub>2</sub>. All cells were routinely tested and found free of mycoplasma. All transfections were performed using a CellPfect Transfection kit (calcium phosphate procedure; Amersham Pharmacia, Piscataway, NJ) according to the manufacture's directions. For Western blot of VP-Rep fusion proteins, 293 cells were transfected with VP22-Rep or Rep-VP22 constructs (or their associated modified constructs lacking a functional NLS) using calcium phosphate coprecipitation. Cells were harvested after 48 hours and lysed. Proteins were separated using SDS-PAGE electrophoresis, and transferred to nitrocellulose. The western analyses demonstrated expression of the AAVREP<sub>490</sub>V22 fusion protein following transfection. See Figure 4.



Please replace paragraph [00046] with the following amended paragraph:

**[00046]** Amino and carboxyl-terminal VP22/AAV2 Rep fusion proteins encoded by expression plasmids were initially tested for their ability to traffic intercellularly after calcium-phosphate transfection into 293 cells. For immunofluorescence assays, approximately  $6.0 \times 10^5$  293 cells were plated on coverslips in 6-well plates and transfected with 1-3  $\mu\text{g}$  of expression vector DNA for the various Rep derivatives. At specified times post-transfection, cells were briefly washed 3 times in room temperature phosphate-buffered saline (PBS), fixed in methanol at  $-20^\circ\text{C}$  for 5 minutes, and permeabilized by incubating them in acetone for 2-5 minutes at  $-20^\circ\text{C}$ . The fixed cells were subsequently blocked with 1% BSA/1x PBS for 5 minutes at room temperature and stained with 1  $\mu\text{M}$  primary mouse monoclonal anti-rep (such as CAT# MAB6030, Maine Biotechnology, Portland, ME) or anti-c-myc antibody (such as CAT# R950-25, Invitrogen) diluted in 1% BSA/1x PBS, for 1 hour. The cells were then washed 3 times in PBS, 5 minutes each time, and incubated for 1 hour with a FITC-conjugated goat anti-mouse IgG secondary antibody (such as CAT# sc-2010; Santa Cruz Biotechnology, Santa Cruz, CA) and DAPI (4',6-diamidino-2-phenylindole; Sigma, St. Louis, MO). Following

the washes, the fixed cells were briefly rinsed in sterile dH<sub>2</sub>O, air-dried, and mounted onto glass slides using a 50% glycerol in dH<sub>2</sub>O. All staining procedures were conducted at room temperature. Cells were photographed by epifluorescence on a Nikon Labophot-2 photomicroscope with fluorescein and DAPI filters using a Nikon Fluor 40x objective. No visible staining of the full length ~~construct~~ RepVP22 protein was seen outside of the nucleus. Therefore, it appears that Rep-NLS overrides VP22's inherent nature to traffic outside of cell.

Please replace paragraph **[00047]** with the following amended paragraph:

**[00047]** The ability of VP22AAV2Rep<sub>491</sub> and VP22(Gly)<sub>4</sub>AAV2Rep<sub>491</sub> ~~constructs~~ proteins to traffic intercellularly also were compared following transfection. Cells were stained for fusion protein with fluorescein isothiocyanate (FITC) and with 4'-6-diamidino-2-phenylindole-2HCl (DAPI) to visualize the cells. See Figure 5. Panels 5A and 5B show immunofluorescent staining indicating the presence of the fusion protein. The results indicate that the 4-glycine insert ~~construct~~ protein traffics intercellularly.

Please replace paragraph [00049] with the following amended paragraph:

**[00049]** An rAAV vector-containing plasmid pCWRHIVAPAP and a nucleic acid encoding one of the relevant Rep derivatives were cotransfected using calcium phosphate into  $1.8 \times 10^6$  293 cells seeded in 60 mm dishes. 293 cells were harvested between 60 and 90 hours post-transfection and washed twice with PBS at 4°C. Cell pellets were suspended in 100 mM NaCl, 25 mM EDTA, and 10 mM Tris, pH 8.0, with 1 µg/ml RNase A and incubated for 2 hours at 37°C. Sodium dodecyl sulfate (SDS) and Proteinase K then were added to a final concentration of 0.5% and 0.1 mg/ml, respectively, and the mixture was incubated overnight at 56°C. Genomic DNA was purified from the digested cell pellet material by phenol/chloroform extraction, followed by ammonium acetate/ethanol precipitation. Isolated DNA was quantified via spectrophotometric analysis. Similar experiments were performed using CWRHIVAPgkH, an rAAV vector encoding resistance to hygromycin. In these experiments, cells were grown in media supplemented with 250 µg/mL hygromycin and 400 µg/mL G418 to select for cells expressing the rAAV vector and fusion protein, respectively. Colonies resistant to both hygromycin and G418

were isolated and expanded. Genomic DNA was extracted from the cell lines as described above.

Please replace paragraph **[00051]** with the following amended paragraph:

**[00051]** To confirm rAAV vector site-specific integration into AAVS1, PCR products corresponding to vector cellular junction sequences were inserted into pGEM-T vectors (Promega, Madison, WI), amplified in DH5 $\alpha$  cells, and subjected to agarose gel sequence analysis in two independent Southern analyses, one probed with an ~~rAAV-rAAV vector~~-specific (Figure 9) and the other with an AAVS1-specific (Figure 10) probe. PCR reactions were performed using the Taq DNA polymerase kit (Qiagen), designed to amplify DNA containing secondary structures following the manufacture's directions. Amplified products were separated using 0.8% agarose gel electrophoresis in duplicate, and transferred overnight to a nitrocellulose membrane according to methods known in the art. After cross-linking the DNA samples to the filter blot, the membrane was cut in half, each half containing a complete set of the samples to be analyzed. One blot half was hybridized with a random primed <sup>32</sup>P-labeled ~~AAVAAV vector~~-specific probe while the other half was hybridized with a

AAVS1-specific probe. Bands that were positive with both probes indicate site-specific integration. Western blots were used to confirm the different sizes of mutants. Phosphorimaging analysis (Molecular Dynamics) was used to evaluate the extent of rAAV vector integration. See Figure 8.

Please delete Tables III-VI, pp. 27-38.